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TITLE: Development of a Novel Methodology for Improving CTL
Recognition of Prostate Specific Antigen (PSA) for the
Immunotherapy of Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) The major goal of the proposed research is to develop a novel methodology for improving T cell epitopes. The underlying hypothesis is that T cells capable of recognizing tumor-associated antigens (TAA) are present but often difficult to activate. However, once activated such T cells might be effective against tumors due to the less stringent triggering requirements of mature effectors. We proposed to develop a novel bacterial expression system for modifying and screening the epitopes of PSA, a known TAA. In the current year we have demonstrated the system devised can discriminate peptides with known differences in their ability to stimulate T cells. Further, we have developed a suitable means for mutagenizing each position in a target peptide and a library encoding altered peptides has been constructed and characterized. An initial screen of this library identified an altered peptide ligand that appears approximately 40 fold better able to stimulate a PSA reactive hybridoma. The findings obtained support the hypothesis that altered peptide ligands can be discovered using the novel methodology that we are developing. The studies to date have established "proof of principle" that this method can be used to improve T cell epitopes.			
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INTRODUCTION

Effective immunotherapy for cancer has long been a goal of tumor immunologists. Cytotoxic T cells (CTL) may effectively lyse tumor cells expressing tumor associated antigens (TAA) if the T cells are initially activated sufficiently since effector T cells have much less stringent activation requirements than naïve T cells. However, T cells are often poorly activated to these tumor-associated antigens. One approach to improving the activation of CTL is to modify the target epitopes so that the T cells are activated more effectively, yet retain activity against the original epitope. A major limitation of this strategy is that it is difficult to test all the possible epitopes in an unbiased fashion. A major goal of the proposal is to develop a novel methodology to improve T cell epitopes of PSA that could be used ultimately in the immunotherapy of prostate cancer.

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- Task 1. Generation of CTL and class I restricted hybridomas (1-18 months)
- Task 2. Identification of class I restricted epitopes (months 1 - 24)
- Task 3. Develop and screen library of mutant epitopes (months 6-30)
- Task 4. Testing and analysis of improved epitopes (months 24-36)

Research Accomplishments associated with the above tasks

Task 1. Generation of CTL and class I restricted hybridomas

Task 2 Identification of class I restricted epitopes

The progress on task 1 and 2 will be discussed in one section because of the close interplay of these two tasks. We have identified one of the major CTL epitopes of PSA (HPQKVTKFML) in BALB/c mice, which we abbreviate HL10, using the PSA reactive hybridoma 1E7. This peptide epitope binds the L^d molecule. Interestingly, others have shown that L^d epitopes generally are somewhat weaker due to looser binding pocket of the Ld molecule (1). This may be beneficial in that it may be possible to enhance the epitope significantly by improving the binding of the peptide to the L^d molecule. It is important to note that the HL10 epitope was initially identified in normal BALB/c mice. In order to determine if HL10 could serve as an epitope in mice that express PSA, PSA transgenic and nontransgenic mice were immunized with the HL10 peptide. Ten days later the mice were sacrificed, the draining lymph nodes harvested, and these cells were restimulated on the lipopolysaccharide (LPS) stimulated B cell blasts pulsed with the HL10 peptide. The responding CTL were then analyzed for their ability to lyse targets pulsed with peptides at varying concentrations. In these experiments, higher avidity T cells would be expected to lyse targets at lower concentrations of peptide. Conversely, lower avidity T cells would require higher concentrations of peptides. An assessment of T cell avidity could be achieved by comparing the level of peptide required to achieve the same level of lysis. Examining several effector to target ratios ensures reproducibility of the phenomenon and gives another measure of the relative avidity of the T cells. Representative results of such assays, using CTL derived from either transgenic or normal mice, are shown in **Figure 1**.

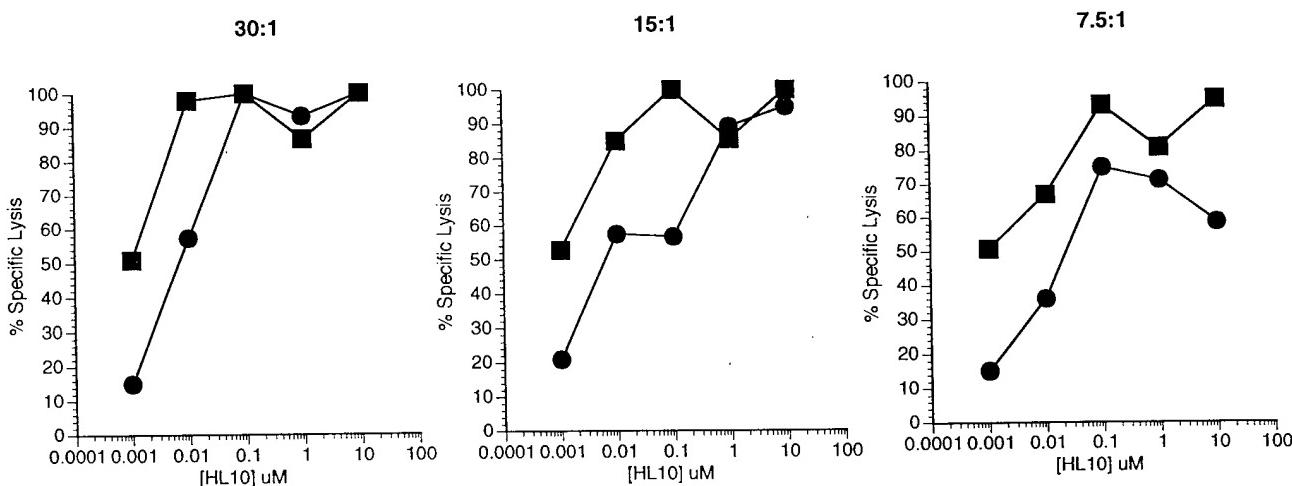


Figure 1. CTL from PSA transgenic mice exhibit CTL responses that are of lower overall avidity. PSA transgenic and non-transgenic mice were immunized with PSA 188-197 (HL10) peptide. Lymph node cells were isolated and restimulated on LPS blasts pulsed with HL10. After 5 days the cells were analyzed by standard Chromium release assay using RMA-S Ld cells pulsed with the indicated concentrations of HL10 as targets. Numbers at the top of each graph indicate the effector to target ratios employed. Squares are T cells from nontransgenic mice whereas circles are T cells derived from transgenic mice.

Several points can be made from these assays. First, T cells from both transgenic as well as nontransgenic immunized mice are able to respond to peptide pulsed targets and do not lyse cells when peptide is absent. As an additional control, the P815 cells were pulsed with an irrelevant peptide derived from the LCMV virus that binds to the Ld molecule, and also tested in these assays. These targets were not lysed appreciably (data not shown). Taken together, these data illustrate the specificity of the response. Second, these data show that T cells from transgenic mice can respond to the HL10 epitope and are not absolutely tolerant to this peptide. Third, there is a difference in the ability of the CTL from transgenic and nontransgenic mice to lyse peptide pulsed targets. In this experiment, there is about 10 to 15-fold difference in the concentration of peptide required to sensitize targets for lysis by CTL from the transgenic mice compared to CTL derived from nontransgenic mice. This is significant since it suggests that while responses can be made in the transgenic animals, they are of lower avidity, and their generation might be improved by modifying the peptide epitope. In summary, these experiments illustrate that CTL responses can be made to a defined epitope of PSA. Further, this defined epitope as well as the class I restricted hybridoma necessary to begin mutagenesis studies as outlined in task 3 are now available.

We also wished to determine if it was possible to generate CTL to other epitopes of PSA. We were able to show that the cytokines IL-12 and IL-2, when present in the tumor microenvironment, helped generate a substantial response to PSA (see figure 2). By analyzing the tumor infiltrating lymphocytes (TIL) we were able to show that these other epitopes could function as targets for T cells. This response appears largely directly toward epitopes other than HL-10 of PSA. While the identification of the HL10 epitope allows us to proceed with subsequent tasks, analysis of another epitope may serve as a useful comparison.

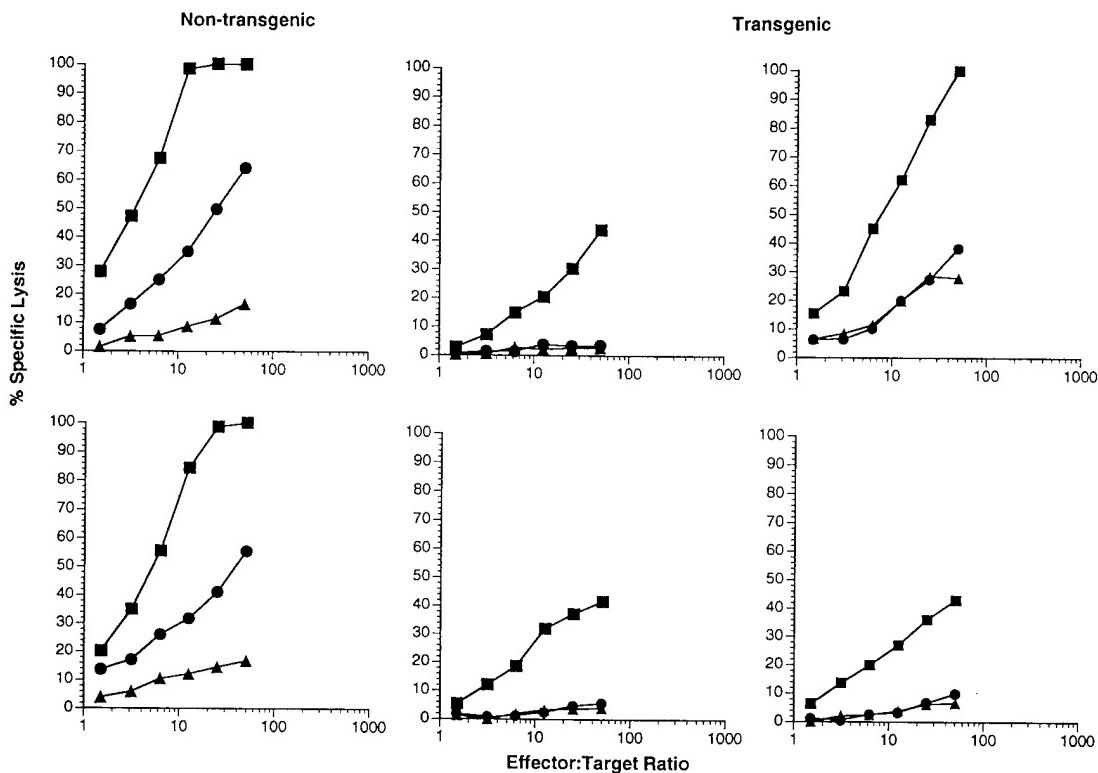


Figure 2. IL-12 and IL-2 induces PSA specific CTL responses in PSA transgenic mice. PSA transgenic and nontransgenic mice were challenged with 1×10^7 Line 1 PSA/IL-2/IL-12 tumor cells. 12 days after challenge, tumor infiltrating lymphocytes (TIL) were isolated and analyzed by standard 6 hour chromium release assays. Targets used were P815 PSA (squares), P815 pulsed with 188-197 (circles), or P815 neo (triangles).

Task 3: Develop and screen library of mutant epitopes

A major portion of our strategy for improving T cell epitopes depends on a screen using T cell hybridomas and their ability to respond to fusion proteins produced in bacteria. In the strategy proposed, the fusion proteins are coupled to beads to facilitate their uptake and incubated with professional antigen presenting cells (APC). These cells take up the beads, process the antigen, and in turn are used to stimulate the T cell hybridomas. This stimulation is assessed by taking advantage of a reporter construct driving β -galactosidase in the T cell hybridoma, which is activated upon T cell stimulation. An important question is whether the assay designed could discriminate peptides with differential ability to stimulate T cells. In order to address this critical question we devised a test system using an epitope of ovalbumin, SIINFEKY_L (SL8). For these experiments, we could take advantage of a well-characterized T cell hybridoma called B3Z, as well as peptides known to stimulate the responding T cells differently. The wild type peptide SL8 stimulates T cells reactive with this epitope very effectively. However, the mutated peptide or altered peptide ligand (APL), in which the lysine (K) at position 7 is mutated to an alanine

(A), can stimulate the cells albeit much less effectively (2). In order to test the methodology proposed, we made constructs encoding the wild type SL8 peptide as well as 7K to 7A mutant using oligonucleotides and standard recombinant techniques. These were expressed as fusion proteins attached to beads and assayed as described above. The results of this experiment are illustrated in Figure 3.

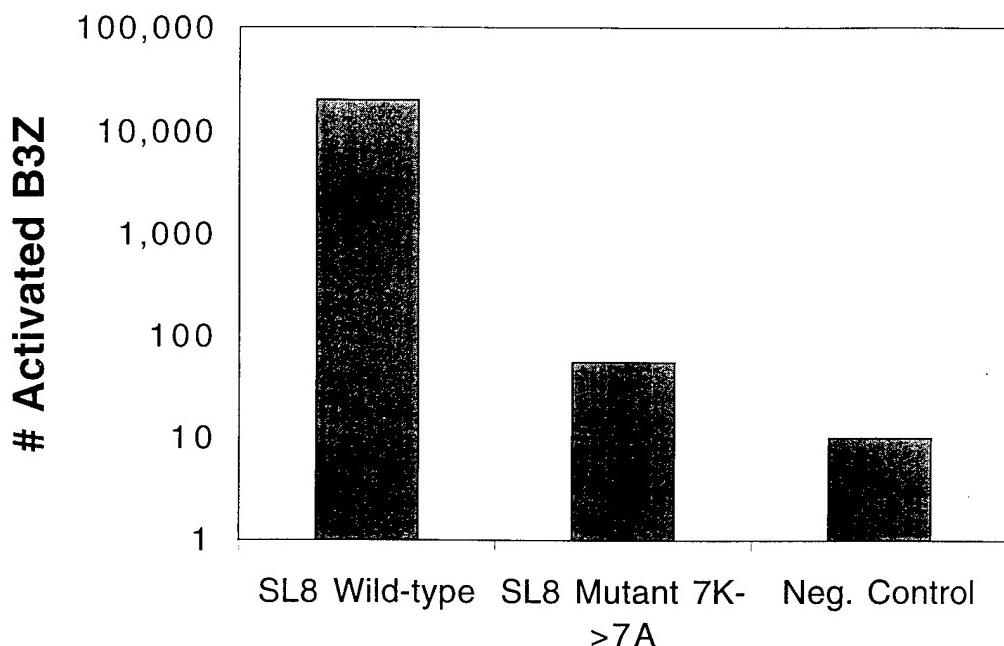


Figure 3: The hybridoma screening system utilizing bacterial fusion proteins can distinguish altered peptide ligands from the wild type peptide. The B3Z hybridoma reactive with the SL8 peptide was tested in an antigen presentation assay using either fusion proteins isolated from bacteria adsorbed to beads encoding either the wild type epitope (SL8) or with a mutant of SL8 in which the lysine at position 7 is changed to an alanine.

As can be seen in Figure 3, the wild type peptide SL8 stimulates very effectively compared to the negative control. In contrast, the altered peptide ligand (APL) of SL8 (7K to 7A mutant) is much less stimulatory. Thus, the screen as outlined is sufficiently robust and can discriminate between peptides with known differences in their ability to stimulate T cells. Note in this case that the APL is less stimulatory than the wild type peptide, the reverse of the proposed experiments for PSA. The key finding in the experiment shown in Figure 3 is that differences in the ability of peptides to stimulate CTL is reflected in the ability of the T cell hybridomas to be stimulated in these assays. This is a critical finding in that it validates the methodology using known reagents and strongly suggested it could be used to identify improved PSA epitopes if they exist.

Generation and characterization of libraries

A major task is to create mutant libraries encompassing known epitopes. The original strategy was to employ saturation mutagenesis techniques to a single library encompassing the entire peptide epitope. The initial approach was to use spiked pools of bases to synthesize oligonucleotides that would encode the desired peptide epitope, which should result in mutations

throughout the entire epitope. This approach was tried using several different levels of spiking in the base pools used to synthesize the oligonucleotides. These were then cloned into expression vectors and the resulting bacteria clones were analyzed by DNA sequencing. The results of these analyses revealed a much lower percentage of mutants than we had anticipated. Several attempts were made using variations of this strategy, all of which were unsuccessful. The reason for this remains uncertain, but because the frequency of mutations was unacceptably low we developed an alternative strategy for creating mutant epitopes. In the new approach, we attempted to create a mutant library for each position in the peptide by randomizing one codon at a time. Thus in the case of the HL10 peptide, we would create 10 libraries, one corresponding to each position in the peptide. While more labor intensive in the construction of the library, this approach also offers some advantages, including the ease of analyzing the library as well as simplifying the screening process. In order to test this approach, we mutagenized the first position (P1) of the HL10 peptide. In these experiments we made a random series of mutations in the oligonucleotides corresponding to position 1 (Histidine) of HL-10. These were cloned and 36 of the resulting colonies were picked, plasmids isolated, and the sequence corresponding to the epitope was determined. A representative sample of the mutations is shown.

Clone #	Mutation (Codon/a.a.)
Wild Type	CAC, HIS
2A	GUU, VAL
2B	GUC, VAL
2C	CGC, ARG
2D	GGG, GLY
3C	CUU, LEU
3D	CAU, HIS
4A	UCC, SER
4B	CUC, LEU
4C	UCC, SER
4D	CGU, ARG
5C	UAU, TYR
5D	GCC, ALA
5E	GCC, ALA
9A	AGC, SER
9B	GGG, GLY
9C	GAU, ASP
9D	CCC, PRO
10B	UCG, SER
10D	UUC, PHE
10E	ACC, THR

Figure 4. Summary of representative mutations from the HL10 position 1 library. Colonies were picked, plasmids isolated, and the DNA sequence of the region encoding the epitope was determined. For convenience, only the amino acid change is shown.

As can be seen, this strategy very effectively generates mutations and can generate mutations that are 3 bases different from the wild type. For example we have a clone in which the wild type sequence CAC (His) is replaced by GUU (Val). As expected, no mutations were detected in positions other than position 1 (data not shown). This approach worked to generate a wide

variety of mutations suitable for screening. Thus we have developed a suitable strategy for generating mutations in the target epitope. Further efforts will be devoted towards making the libraries at subsequent positions (i.e. positions 2-10 of HL-10).

Screening of P1 library

The position 1 library was screened using the 1E7 hybridoma that reacts with the HL-10 peptide derived from PSA. In this screen, a bacterial colony containing an expression plasmid encoding the wild type peptide epitope was included as a positive control. As can be seen in Figure 5, most of the bacterial clones stimulate very similarly to the wild type. A few appeared very stimulatory (e.g. 5D, 5E, 10A).

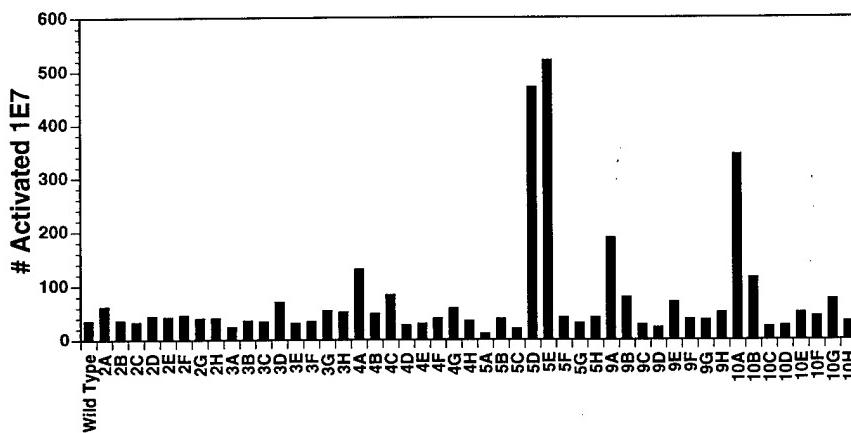


Figure 5: HL10 mutant constructs were screened for the ability to stimulate an H2-L^d restricted CTL hybridoma that is specific for wild type HL10. Recombinant HL10 mutant protein constructs were bound to beads and introduced into cultures containing an H2-L^d expressing antigen presenting cell line, RAW, along with an HL10 specific CTL hybrid, 1E7. 1E7 contains the β -gal gene under the control of the IL-2 promoter. 1E7 activation was determined by the number of β -gal expressing cells/ 2×10^5 1E7, using the hydrolysis of the substrate x-gal to visualize the cells.

Interestingly, the clones which gave the most stimulation encoded the same change (H to A). Finding independent colonies encoding the same change that stimulated the hybridoma approximately 10 fold better than the wild type suggested the screen was indeed working. Based on these results we synthesized the peptide AL-10. The AL-10 peptide was then tested under defined concentrations and compared to the wild type HL-10 peptide for its ability to stimulate 1E7. The results of this comparison are shown in figure 6.

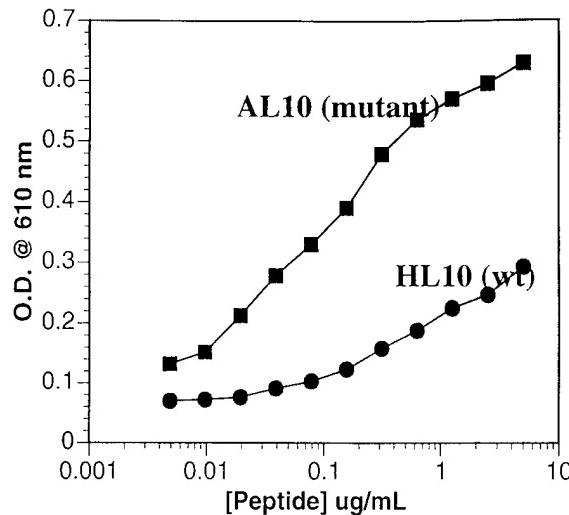


Figure 6: Comparison of the ability of the HL10 and AL10 peptides ability to stimulate the 1E7 PSA reactive hybridoma. The indicated concentrations of the synthetic peptides HL10 and AL10 were incubated with the 1E7 hybridoma and RAW cells as APC. The amount of activation was determined using the soluble beta galactosidase substrate CPRG. Absorbance was measured at 610nm. Note that the assay is similar to that for the initial screen except that T cell hybridoma activation was assessed using a soluble substrate for beta galactosidase rather than the insoluble substrate X-gal

As can be seen figure 6, the AL10 peptide stimulates the 1E7 hybridoma at much lower concentrations than the HL10 peptide. In this experiment this difference is approximately 40 fold. These data strongly show that we have identified an altered peptide ligand with increased stimulatory activity. These data also validate the overall methodology.

Task 4 Testing and analysis of improved epitopes – this task remains to be initiated (per the original Statement of Work).

KEY RESEARCH ACCOMPLISHMENTS

- Generation of CTL and demonstration that CTL can recognize the HL10 epitope
- Development of a methodology for generating a mutant library
- Generation and characterization of a position 1 library of HL10
- Demonstration of feasibility of proposed screening technique
- Initial functional analysis of the HL10 P1 library
- Identification of an altered peptide ligand resulting in increased activation of a PSA reactive hybridoma
- Confirmation of enhanced activation using a synthetic peptide

REPORTABLE OUTOMES

Manuscripts, abstracts, presentations:

Turner, M., C. Abdul-Alim C., Willis, R., Fisher, T., Lord, E., Barth, R., and Frelinger J.T Cell Antigen Discovery (T-CAD) assay: A novel technique for identifying T cell epitopes. (Manuscript in preparation).

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Ms. Andrea Brooks was provided (Ms. Brooks is a recent college graduate, who has been working on this project as a laboratory technician). It is anticipated that Ms. Brooks will apply to graduate school within the next 2-3 years, to obtain her Ph.D. and the present research experiences should assist her in that goal. Advanced research training for Dr. Nocera, a postdoctoral fellow in the laboratory, was provided. This experience should substantially broaden her training and experience in cancer research.

CONCLUSIONS

The specific conclusions that can be drawn from the first year of experiments are as follows:

1. CTL can be generated in transgenic mice expressing PSA and the CTL which recognize the HL10 epitope from transgenic mice appear to be of lower avidity.
2. The screen devised can discriminate peptides with known differences in their ability to stimulate T cells.
3. An efficient means of generating mutations has been developed in which each amino acid of a peptide is mutagenized.
4. A screen of the P1 HL10 library identified an altered peptide ligand that appears 40 fold better able to stimulate the PSA reactive hybrid 1E7

The findings obtained support the hypothesis that altered peptide ligands can be discovered using the novel methodology that we are developing. We have shown that the system designed can distinguish known epitopes with different abilities to activate T cells. Using this method, we have also identified an altered peptide with an increased ability to stimulate a T cell hybridoma. The studies to date have established "proof of principle" that this method can be used to improve T cell epitopes of PSA. This methodology should be applicable to other tumor antigens.

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Appendix: None